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Title: Development and characterisation of a novel three-dimensional interkingdom wound biofilm model

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Abstract

Chronic diabetic foot ulcers are frequently colonised and infected by polymicrobial biofilms that ultimately prevents healing. In this study, we aimed to create a novel *in vitro* inter-kingdom wound biofilm model on complex hydrogel-based cellulose substrates to test commonly used topical wound treatments. Inter-kingdom triadic biofilms composed of *Candida albicans*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* were shown to be quantitatively greater in this model compared to a simple substrate when assessed by conventional culture, metabolic dye and live dead qPCR. These biofilms were both structurally complex and compositionally dynamic in response to topical therapy, so when treated with either chlorhexidine or povidone iodine principal component analysis revealed that the 3-D cellulose model was minimally impacted compared to the simple substrate model. This study highlights the importance of biofilm substrate and inclusion of relevant polymicrobial and inter-kingdom components, as these impact penetration and efficacy of topical antiseptics.

Background

Chronic wounds are associated with unacceptably high morbidity and mortality rates, in addition to being a significant economic burden to the National Health Service (NHS) in the UK. It is estimated that the NHS spends in the region of £900 million per year on diabetic foot ulcer treatments and resultant amputations (Hex et al. 2012). Infection risk is one of the overriding factors driving these costs and complications, and the capacity of endogenous and exogenous microorganisms to form complex biofilms within these compromised skin environments hinders chemotherapeutic management (Alavi et al. 2014).

Pathogenic biofilms are frequently associated with chronic wounds (James et al. 2008, Neut et al. 2011). These structures complicate treatment strategies due to enhanced adaptive resistance profiles, primarily driven by the physical composition, including the production of extracellular matrix (ECM) that prevents diffusion of antimicrobials into the biofilm (Davies 2003, Pozo and Patel 2007, Ramage et al. 2012a). ECM-associated degradative enzymes, hypermutability, and persister cells, are just a few examples of how these complex communities survive and adapt to antimicrobial challenge (Davies 2003, Høiby et al. 2010, Pozo and Patel 2007, Ramage, et al. 2012a). Complicating chemotherapeutic intervention even further is the polymicrobial nature of the biofilms found in diabetic foot ulcers (Smith et al. 2016). Moreover, increasing evidence that yeasts and moulds play important contributory roles in exacerbating infections suggests inter-kingdom biofilms deserve consideration (Peters et al. 2012).

S. aureus and *P. aeruginosa* are the two most frequently isolated bacterial species from such chronic and difficult-to-treat biofilm infections (Citron et al. 2007, Hartemann‐Heurtier et al. 2004, MacDonald et al. 2002). They are often co-isolated and are associated geographically within the wound site (Fazli et al. 2009). In contrast to bacteria, despite reports their importance, the role of pathogenic fungi in wound biofilms, are relatively under-investigated and underappreciated clinical entities (Appelgren et al. 2002, Dowd et al. 2011, MacDonald, et al. 2002, Santucci et al. 2003, Sun 2010, Weinstein and Mayhall 2003). *Candida* species are the primary fungal pathogen isolated from these

infections, although this organism rarely colonises healthy intact skin (Grice and Segre 2011). Several models have been described in recent years that have examined these paradigm nosocomial pathogens in triadic systems (Hoekstra et al. 2016, Kart et al. 2014). Though a caveat to the utility and translation of these models is the basic 2-dimensional nature of the substrates used, which are not at all representative of a wound environment (Hill et al. 2010, Hoekstra, et al. 2016, Kart, et al. 2014). The development of cellulose matrix based models supported by hydrogels that better mimic the consistency of the wound surface enables biofilms to form in a 3-dimensional matrix. Using either poloxamer, collagen, or agarose hydrogel, a complex hydrated structure is formed which induces the development of the biofilm phenotype (Clutterbuck et al. 2007, Harrison et al. 2015, Percival et al. 2007, Strathmann et al. 2000). The main applicability and translation usefulness of these model systems lies in their utility in the development and testing of antimicrobial anti-biofilm molecules. To date, these models have tended to focus on mono-species biofilms.

Systemic antibiotics are commonly used to treat chronic wounds, yet there is controversy over their usage and rising concerns over the development of antimicrobial resistant organisms (Atiyeh et al. 2009, O'meara et al. 2001). Topical wound washes and ointments are often recommended as an alternative to, or in combination with, systemic treatment (Atiyeh, et al. 2009, Snell et al. 2013). Chlorhexidine (CHX) and povidone iodine (PVP-I) are two of the most commonly used clinically due to their high levels of biocidal activity (Atiyeh, et al. 2009, O'meara, et al. 2001).

The aims of the present study were therefore to develop a polymicrobial inter-kingdom *in vitro* biofilm model on complex substrates that can be used to test clinically relevant antimicrobial therapeutics. Here we show for the first time the use of a novel biofilm substrate that can be adapted to represent a wide variety of wound infection biofilms, and its application for chronic wound biofilm research.

Methods

Culture conditions and standardisation

A selection of characterised laboratory strains were used in this study, including the bacteria *P. aeruginosa* PA14 (Rahme et al. 1995), *S. aureus* Newman's strain (Duthie and Lorenz 1952) and the yeast *Candida albicans* SC5314 (Fonzi and Irwin 1993). Both bacteria were grown and maintained at 37°C on Luria agar (Sigma-Aldrich, Dorset, UK), while *C. albicans* was grown and maintained at 30°C on Sabouraud dextrose agar (SAB [Sigma-Aldrich, Dorset, UK]). All isolates were stored indefinitely in Microbank® vials (Pro-Lab Diagnostics, Cheshire, UK) at -80°C.

Overnight broths of *P. aeruginosa* and *S. aureus* were prepared in 10 mL Luria broth ([LB] Sigma-Aldrich, Dorset, UK) at 37°C and *C. albicans* was propagated in 10 mL yeast peptone dextrose broth (YPD [Sigma-Aldrich, Dorset, UK]) at 30°C at 150 rpm. Overnight cultures were washed twice by centrifugation (1600 x *g*) and resuspended in 10 mL phosphate buffered saline (PBS). All cultures were standardised and adjusted to 1×10^8 cells/mL, using optical density at 590 nm for bacterial strains and a haemocytometer for *C. albicans*.

Hydrogel preparation

Hydrogels were composed of 10% 3-sulfopropyl acrylate potassium salt, 0.95% v/v poly(ethylene glycol) deacrylate (PEG), 0.01% v/v 1- hydroxycyclohexyl phenyl ketone, with the addition of 50% heat-inactivated horse serum ([HS] Thermo Fisher Scientific, Loughborough, UK) in sterile water to the final volume. To a 12-well flat-bottomed microtiter plates (Corning Incorporated, NY, USA), 2 mL of hydrogel was added to each well before being polymerised under a 366 nm ultraviolet (UV) Lamp (Camag, Hungerford, UK) for 30 min within a class II laminar flow hood. These polymerised hydrogels were then stored at 4°C until required, for up to one week.

Biofilm development and antimicrobial therapy

All organisms were standardised to a final working concentration of 1×10^6 cells/mL in 50% v/v HS (Life Technologies, Paisley, UK) for biofilm development. For viability and biomass assays (described below), 200 µL of single species

and triadic species suspensions were added to 96-well flat-bottomed microtiter plates (Corning Incorporated, NY, USA). For quantitative polymerase chain reaction (qPCR) and viable cell counting, 500 µL of cultures were added to Thermanox™ coverslips (13 mm diameter, Fisher Scientific) contained within 24 well plates (Corning, NY, USA). Biofilms were incubated at 37°C for 24 h to develop. All procedures were carried out in a class II laminar flow hood. For biofilm development on hydrogels, organisms were standardised to a 1×10^6 cells/mL in PBS and added to sections of cellulose matrix (1.25 cm^2) (IPS Converters, Oldham, UK). Following initial incubation at 37°C with agitation for 2 h, the matrix was then placed on top of the hydrogel surface and incubated at 37°C for 24 h. Negative controls containing no inoculum were also included. All testing was carried out in triplicate, on three separate occasions. Following biofilm development, cells were washed twice with PBS to remove any non-adherent cells before treatment with 10% w/v PVP-I (Sigma) or 0.05% v/v CHX (Sigma) for a further 24 h at 37°C. Untreated controls were also included.

Assessment of treatment using conventional quantitative culture

To assess the viability of the organisms contained within the biofilm, viable cell counting was performed. Following treatment, biofilms were sonicated in 1 mL PBS, from Thermanox™ coverslips or hydrogel cellulose matrix at 35 kHz for 10 min to remove the biomass, as described previously (Ramage et al. 2012b), prior to the Miles and Misra technique (Miles et al. 1938). Decimal serial dilutions were plated on LB and SAB agar, which were incubated at 37°C and 30°C, respectively for 72 h for Thermanox™ coverslips, and 48 h for hydrogel. The number of colonies were counted and represented as total bacteria and total yeast colony forming units (CFU) per mL.

Assessment of treatments using quantitative viability assays

Following treatment, biofilms were washed twice with PBS before biofilm viability and biomass were quantified. Viability was assessed by the AlamarBlue® assay (Invitrogen, Paisley, UK), as per manufacturer's instructions (Kirchner et al.

2012). Absorbance was measured spectrophotometrically at 570 nm and the reference wavelength at 600 nm (FluoStar Omega, BMG Labtech). All assays were performed in triplicate, on three separate occasions.

Assessment of treatment using live/dead quantitative PCR

Viability based qPCR, a technique shown to differentiate between viable and dead cells (Alvarez et al. 2013, Sanchez et al. 2013, Sanchez et al. 2014, Sherry et al. 2016), was used to assess the composition and viability of the biofilms at a molecular level. Samples were prepared as previously described by our group using propidium monoazide (PMA), a DNA intercalating dye, which prevents DNA from cells with compromised membranes from being detected by PCR (Sherry, et al. 2016). Briefly, biofilms were sonicated from Thermanox™ coverslips or cellulose matrix, 50 µM of PMA was added to each sample before incubation in the dark for 10 min to allow dye uptake. To permit binding of the PMA, samples were exposed to a 650 W halogen light for 5 min. DNA was then extracted using the QIAamp DNA mini kit, as per manufacturer's instructions (Qiagen, Crawley, UK). Controls containing no PMA were also included for each sample to determine total biomass.

Following DNA extraction, qPCR was used to enumerate both the live and total cells of each species remaining in the biofilm following each treatment. In brief, 1 µL of extracted DNA was added to a mastermix which contained 10 µL Fast SYBR® Green Master Mix (Life Technologies, Paisley, UK), 7 µL water and 1 µL of 10 µM forward and reverse primers for each bacterial or fungal species. Primer sequences are shown in Table 1. The thermal profile of 95°C for 20 s followed by 40 cycles of 3 s at 95°C, and 30 s at 60°C was used in this study. Three independent replicates for each treatment were analysed in duplicate using Step One Real-Time PCR system and software (Life Technologies, Paisley, UK). Samples were quantified to calculate the colony forming equivalent (CFE) based upon a standard curve per reaction performed.

Scanning electron microscopy

Biofilms were grown on Thermanox™ coverslips or hydrogel cellulose matrix and treated, as previously described. Biofilms were washed twice with PBS, before being fixed in 2% para-formaldehyde, 2% glutaraldehyde, 0.15M sodium cacodylate, and 0.15% w/v alcian blue, at pH 7.4, and prepared for SEM as previously described (Erlandsen et al. 2004). The specimens were sputter-coated with gold and viewed under a JEOL JSM-6400 scanning electron microscope.

Statistical analysis

Graph production, data distribution and statistical analysis were performed using GraphPad Prism (version 6; La Jolla, CA, USA). Unpaired t-tests were used to establish significant differences between treatments and substrate types for viability assay scores and CFEs. Percentage viability scores were log transformed before statistical analysis took place. For conventional quantitative culture, Mann-Whitney test was used. Statistical significance was achieved if $P<0.05$. Next, viable composition datasets were reduced by $\log_2$ transformation so as to carry out principal component analysis (PCA) using PAST software (Hammer O 2001). A scree plot was used to determine how many components emerged. To determine if statistically distinct clusters formed on the PCA plots, new variables were created for each principle component by using the factor loadings as regression coefficients, producing a score for each sample. These scores were then used as outcome variables to compare between groups using an unpaired t-test.

Results

Standard 2-D biofilm models show antibacterial agent efficacy in mono-species, while triadic biofilms support some resistance

Firstly, we wanted to establish a baseline using a typical substrate used for *in vitro* biofilm studies, i.e. a 2-D polystyrene model. Here we tested two key topical agents using conventional culture, and based upon this methodology PVP-I was shown to be the most effective treatment, completely eradicating the bacterial and yeast biofilm burden of all mono-cultures ($P<0.0001$) (Figure 1A). In addition, CHX was equally active against *P. aeruginosa* mono-culture biofilms and bacteria in the triadic species biofilms, significantly reducing total bacterial counts by >6 $\log_{10}$ (Figures 1A and 1B). *S. aureus* and *C. albicans* mono-species biofilms were also reduced by CHX treatment (Figure 1A), but only by >2 $\log_{10}$ ($P<0.05$, $P<0.001$, respectively). However, in the 2-D triadic model yeast counts were completely unaffected (Figure 1B). Using soluble metabolic dyes (Figure 1C), in the 2-D model *C. albicans* and *P. aeruginosa* mono-culture viability was significantly reduced by both PVP-I and CHX ($P<0.0001$). CHX was able to significantly inhibit *S. aureus* biofilms ($P<0.05$), but PVP-I showed no significant reduction. In contrast, in the triadic culture both CHX and PVP-I caused significant decreases in viability ($P<0.0001$).

Molecular analysis reveals reservoirs of viable cells remain after treatment of 2-D mono-species and triadic biofilms

The techniques described above are subjective in terms of species-specific quantification, so given these limitations we decided to employ a molecular approach, enabling determination of the precise viable composition of biofilms following active exposure. Despite culture and metabolic evaluation showing a significant reduction in the viability of all biofilms, viable quantitative analysis by qPCR revealed a significant number of cells are retained within each biofilm (Figure 2 and 3). Molecular analysis consistently showed higher reductions with PVP-I compared to CHX treatment in both substrates tested.

C. albicans mono-species total cell count was significantly reduced with both PVP-I ($P<0.0001$) and CHX ($P<0.001$) treatment (Figure 2A). The number of live cells remaining within those treated biofilms was 13% ($P<0.0001$) and 23% ($P<0.01$), respectively. *S. aureus* mono-species total cell count was also significantly reduced (88.5%) with PVP-I ($P<0.0001$), with only 2% live cells remaining ($P<0.0001$) (Figure 2B). CHX treatment, however, showed no difference to the control for both total and live cells ($P>0.05$). *P. aeruginosa* mono-species biofilms were also affected by the two treatments (Figure 2C). *P. aeruginosa* mono-species total cells were significantly reduced by PVP-I (77%, $P<0.01$), and live cells reduced by 98% ($P<0.05$). CHX treatment appeared to cause a significant increase in total cell count ($P<0.01$), though a slight decrease in live cells ($P>0.05$).

The triadic species biofilms were again more effectively treated by PVP-I compared to CHX (Figure 3). The total cell count on the 2-D model was significantly reduced by PVP-I (92%, $P<0.0001$; Figure 3B). Live cells were similarly reduced compared to untreated biofilms (98%, $P<0.001$; Figure 3A). CHX caused a significant increase in total cells, rising by ~2.5 times ($P<0.0001$), whereas live cells marginally decreased (20%, $P>0.05$; Figure 3C).

SEM analysis was used to analyse the biofilm architecture ± treatment (Figure 3). In the 2-D model *C. albicans* was mainly hyphae, acting as a scaffold to which the bacteria tended to co-aggregate upon (see insert magnification). The cell density within the biofilm was reduced by both treatments, although more so with PVP-I.

3-D substrates support culturably greater quantities of mono-species and triadic biofilm cells with enhanced resistance to CHX and PVP-I

Using conventional culture, the 3-D cellulose matrix model, with PVP-I treatment (Figure 4A) significantly reduced *C. albicans*, *S. aureus*, and *P. aeruginosa* monocultures ($P<0.0001$), whereas CHX was ineffective for both *C. albicans* and *P. aeruginosa* ($P>0.05$). *S. aureus*, however, was significantly decreased by CHX ($P<0.0001$; Figure 4A). In the triadic hydrogel model, yeasts and bacteria

were significantly reduced by PVP-I ($P<0.0001$), but not CHX ($P>0.05$; Figure 4B). The metabolic assays confirmed these results (Figure 4C), with both *C. albicans* and *S. aureus* monocultures were significantly reduced by the two treatments ($P<0.0001$). *P. aeruginosa* and triadic cellulose matrix biofilms, although both significantly reduced with PVP-I treatment ($P<0.0001$), were seemingly unaffected by CHX treatment, ($P>0.05$).

Molecular analysis shows 3-D substrates support greater total and viable quantities of mono-and triadic species biofilm cells with enhanced resistance to CHX and PVP-I

Overall, the treatments on 3-D cellulose matrix mono-species biofilms showed similar efficacy patterns to the 2-D model, although, 3-D cellulose matrix models generally had higher numbers of total and viable cells detected by qPCR ($P<0.05$) (Figure 5). *C. albicans* mono-culture total cell count was reduced by 72% by PVP-I ($P<0.01$), whereas CHX was less effective with a 28% reduction ($P>0.05$) (Figure 5A). The live cell count within these biofilms was significantly reduced by 98% and 61% after PVP-I ($P<0.001$) and CHX treatment ($P<0.05$), respectively. Both total and live cell counts for *S. aureus* were reduced by approximately 88% by PVP-I ($P<0.001$) and 98% ($P<0.05$), respectively (Figure 5B). Treatment with CHX was ineffective for total and live cells ($P>0.05$). CHX reduced *P. aeruginosa* total cells by only 15%, and viable cells were unaffected ($P>0.05$) (Figure 5C). PVP-I, however, was significantly effective reducing total cell count by 84% ($P<0.001$) and live cell count by 95% ($P<0.001$).

The triadic 3-D model showed a total cell count reduction by 94% with PVP-I treatment ($P>0.05$; Figure 6B), and 70% with CHX ($P>0.05$; Figure 6C). Viable cell counts were reduced further by PVP-I (97%, $P<0.001$), whilst CHX was less effective (22%, $P>0.05$).

Based the SEM images, it can be clearly shown that there is an increased cell number on the 3-D substrates, irrespective of treatment. The fibrous nature of the cellulose matrix creates a greater surface area. Interestingly, in the 3-D model *C. albicans* is observed mainly as yeast cells, which is reflected in the

viable cell numbers reported above. However, on the 3-D substrate the dominant morphotype was suggestive of *P. aeruginosa*.

Statistical analysis reveals significant differences between treatments and biofilm substrates

The 3-D model was shown to consistently support significantly greater quantities of cells than that of the 2-D model. This was shown by both conventional culture and viability assays ($P<0.01$), and further confirmed by molecular analysis ($P<0.001$). There were some compositional differences in the triadic model revealed by live/dead qPCR, where both *S. aureus* and *C. albicans* decreased in the 3-D model ($P<0.0001$, $P<0.05$, respectively). SEM provided further evidence that irrespective of treatment and substrate, a significant level of biomass is retained, though notably more upon the cellulose 3-D matrix.

Further to this, there were also significant differences observed between the two models after treatment. Conventional culture, viability assays, and molecular analysis revealed that CHX was less effective in the cellulose matrix model ($P<0.05$). PVP-I was similarly effective in both models, with higher recalcitrance observed in the 3-D model for *P. aeruginosa* ($P<0.05$), and the bacterial component of the triadic biofilm ($P<0.01$). Proportional composition differences in the 3-D triadic biofilm were observed after both after CHX ($P<0.0001$) and PVP-I treatment ($P<0.05$).

PCA was used to reduce the dimensionality of the viable cell data, and allowed the data to be plotted along two principal components (Figure 7). Four distinct clusters emerged; cluster 1 containing only untreated 2-D model; cluster 2 was only PVP-I treated 2-D model; cluster 3 contained CHX treated 2-D model and PVP-I treated 3-D model; cluster 4 containing untreated and CHX treated 3-D model. These clusters were statistically distinct ($P<0.05$). Untreated samples scored higher on PC1 (x axis), this is also true of CHX treated 3-D model, which were indistinct from their untreated counterpart. Treated samples generally scored lower on PC1, which is shown by the directionality arrows. 3-D model biofilms generally scored higher on PC2 (y axis), with the exception of CHX

treated 2-D biofilms within cluster 1. Collectively, these data show that 2-D models undergoing treatment can reveal clear effects from antimicrobial challenge, whereas 3-D models are not subject to the same extent of dynamic change.

Discussion

This study set out to test clinically relevant treatments on a newly developed *in vitro* inter-kingdom triadic biofilm model that is more representative of the physical environment and microbial composition of wounds infections. Based on the methods employed, we report that our new developed wound model supports significantly greater quantities of microorganisms, and that this improved structure reduces the effectiveness of widely used topical antimicrobial agents. Overall, irrespective of the model used, PVP-I treatment was generally more effective than CHX in reducing bacterial, fungal and inter-kingdom bioburden. This highlights the need to better understand the biofilm environment, in particular the importance of mono-, multi-species, or indeed inter-kingdom biofilms in these infections.

Models that recapitulate complex biofilm related diseases and test antimicrobial agents are difficult. Moreover, the ways in which these models are interrogated to generate meaningful data are often flawed. The use of conventional plate counting is still wide spread despite the inherent bias towards the outcome antimicrobial challenge. Innovative molecular based methodologies that analyse viability tend to yield data that is accurate, both qualitatively and quantitatively. Apparent “complete and efficient killing” phenomenon demonstrated by conventional microbiological studies of wound biofilms are not unusual (Hill, et al. 2010, Kart, et al. 2014), including on the agents tested here on methicillin resistant *S. aureus-C. albicans* co-cultures and *P. aeruginosa* only biofilms (Hoekstra, et al. 2016). However, molecular viability analysis can often reveal a larger viable population of cells remaining after treatment (Sherry, et al. 2016). This can be expected, given the nature of the extracellular matrix combined with viable but non-culturable (VBNC), or persister cells, which occur naturally within microbial communities.

The novel *in vitro* interkingdom biofilm model characterised herein consistently showed higher cell counts and less effectiveness of the topical agents used compared with biofilms on polystyrene substrates. This may be partially due to the increased surface area within the cellulose matrix of the novel model. The comparison against the standardised plastic substrate showed that although

they are extensively used for many applications (Capita et al. 2014, Kart, et al. 2014, Mottola et al. 2016, Naparstek et al. 2014, Santos et al. 2016), they are not fully representative of the *in vivo* situation. It has been noted previously that organisms grown with the support of hydrogel matrices are less susceptible to antimicrobial treatments (Clutterbuck, et al. 2007, Percival, et al. 2007). Within 3-D structures such as these it has previously been noted that varying metabolic states naturally exist, due to gradients of both oxygen and nutrients (Rani et al. 2007). These gradients are thought to contribute to tolerance of antimicrobials in biofilms, which could also contribute to the effects seen here. This is indeed a limitation of widespread 2-D models. In our study, this was especially evident with the CHX treatment; which although it apparently effective in the 2-D model showed only a minimal effect on the 3-D matrix probably mainly due to the high levels of *P. aeruginosa* within this model, which has been found to be resistant to CHX at the wound wash concentration (Salami et al. 2006). Interestingly, taking a PCA approach we showed that the untreated and CHX treated cellulose models clustered together, indicating there is little change in viable composition, which is reinforced by the other results described herein. While the proportional make-up of the 2-D model was roughly equally split between the three species, in the 3-D cellulose matrix model *C. albicans* and *P. aeruginosa* dominated. *S. aureus* was present in the cellulose matrix model at approximately $2 \times \log_{10}$ lower, which is also reflected in SEM imaging. Conversely, *P. aeruginosa* can be observed covering the 3-D mesh of the untreated cellulose matrix.

Innovative molecular based methodologies that analyse viability tend to yield data that is accurate, both qualitatively and quantitatively. These have been used to scrutinise and evaluate the impact of treatment of wound infections, and are well described. Early wound model studies used qPCR to investigate compositional changes within their chronic wound biofilm model (Dowd et al. 2009). PCR has previously been criticised for being too sensitive and overestimating the population when compared to culture techniques, with this being attributed to eDNA and the presence of dead cells (Castillo et al. 2006, He and Jiang 2005). As table 2 illustrates, live/dead PCR is the most expensive technique used in this work. Although qPCR approaches are more expensive overall, these methods eliminate the subjectivity and non-specificity that are

associated with conventional microbiology approaches. Moreover, detrimental interactions produced by *P. aeruginosa* phenazines have detriment effects on hyphal growth and viability, effects difficult to decipher with conventional approaches (Hogan and Kolter 2002). With our approach, significant differences were demonstrated here in viability, however there was little difference seen between the biomass of untreated and treated biofilms (data not shown), which is consistent with other studies where CHX and PVP-I did not reduce biomass (Sherry et al. 2013, Tote et al. 2010).

No conclusive clinical studies exist which confirm the effectiveness of CHX in either diabetic foot ulcer or chronic wound infection. A study using a bioreactor to form an *in vitro* multi-species biofilm incorporating *Klebsiella pneumoniae, P. aeruginosa, S. aureus* and *Enterococcus faecalis* concluded that the effectiveness of CHX in controlling a pre-formed biofilm may be limited, especially on multi-species biofilms (Touzel et al. 2016).

Similarly, definitive clinical studies for PVP-I are lacking. A recent rat model study found that *P. aeruginosa* infected wounds irrigated with PVP-I had reduced bacterial counts both on the wound surface and within the tissue compared with irrigation with saline (Kanno et al. 2016). However, this model may be more relevant to skin preparation prior to surgery than to the management of chronic wounds. A Cochrane review of the use of antiseptics in pressure ulcers included PVP-I, but not CHX. The study concluded that the relative effects of systemic and topical antimicrobial treatments on pressure ulcers are not clear but the evidence was graded from moderate to low quality (Norman et al. 2015). More useful is the Cochrane review of antibiotics and antiseptics for venous leg ulcers, which concludes that while some evidence supports the use of cadexomer iodine more evidence is required before conclusions can be drawn about the effectiveness of PVP-I or CHX in healing venous leg ulceration (O'Meara et al. 2013).

Conclusions

This study highlights importance creating a polymicrobial *in vitro* biofilm reflective of the microflora of wounds, containing both fungal and bacterial components. Representative 3-D biofilm substrates showed an increased resistance to antimicrobial wound washes compared to the 2-D plastic surfaces.

Indeed, PCA analysis was clearly able to discern how the models reacted to different treatments. The necessity of using multiple viability techniques to analyse different aspects of the biofilm is also recognised. Singular approaches often only analyse one aspect of the biofilm, but by combining techniques multiple outputs can be measured and analysed collectively. In practical terms, this study shows that our ability to influence wound infections of a polymicrobial and inter-kingdom nature are limited with simple treatments, particularly given the resilient capacity of complex biofilms and their potential to remain and seeding reservoirs. Further studies will be important in trying to maximise the removal and decontamination of complex wound infections, potentially reducing patient morbidity and mortality.

Competing interests

None of the authors have any competing interests.

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Figure 1 – Antimicrobial wound washes exhibit cidal activity against polymicrobial 2-D biofilms. Bacterial and fungal biofilms were grown as mono (A) and triadic cultures (B) in a 2-D model, as previously described. Following development, biofilms were washed and treated with PVP-I (10%) or CHX (0.05%) for 24 h. Monospecies (A) and triadic biofilms (B) were assessed by CFU. Viability was also assessed by the alamarBlue® viability assay (C). All testing was carried out in triplicate, on three separate occasions. Data represents mean ± SD, statistical analysis compared untreated to treated biofilms (*p<0.05, **p<0.01, ***p<0.001). #Indicates no cell growth.

Figure 2 – Molecular analysis demonstrates a significant microbial burden remains within 2-D biofilms following treatment. Bacterial and fungal monospecies biofilms were grown in a 2-D model, as previously described. Following development, biofilms were washed and treated with PVP-I (10%) or CHX (0.05%) for 24 h. Live/Dead PCR was performed and colony-forming equivalents (CFE) were calculated from standard curves for *C. albicans* (A), *S. aureus* (B) and *P. aeruginosa* (C). Data represents mean ± SD. * Represents statistical difference in total CFE values and # represents significant differences between live CFE values (*p<0.05, **p<0.01, ***p<0.001). Data represents CFE values calculated from triplicates carried out on three separate occasions.

Figure 3 – Molecular analysis gives insight into cell death in triadic 2-D biofilms, while SEM reveals complex communities in the triadic model. Bacterial and fungal triadic biofilms were grown in a 2-D model, as previously described. Following development, biofilms were washed and treated with PVP-I (10%) or CHX (0.05%) for 24 h. Live/Dead PCR was performed and colony-forming equivalents (CFE) were calculated from standard curves for untreated (A), PVP-I (B) and CHX (C). Data represents percentage composition calculated from CFE values from triplicates carried out on three separate occasions. SEM, shown in lower panels, was conducted as described in the methods. Note *P. aeruginosa* indicated by a solid white arrow, *S. aureus* by white arrow head, *C. albicans* by a black arrow. *C. albicans* is present in predominantly hyphae form; the bacteria can be seen attached to the hyphae. Bars represent 20 µm on lower magnifications (×1000) and 2 µm at higher magnification (inset, ×6000).

Figure 4 – PVP-I shows superior killing activity over CHX on 3-D biofilms. Bacterial and fungal biofilms were grown as mono (A) and triadic cultures (B) in the 3-D model, as previously described. Following development, biofilms were washed and treated with

PVP-I (10%) or CHX (0.05%) for 24 h. Monospecies (A) and triadic biofilms (B) were assessed by CFU. Viability was also assessed by the alamarBlue® viability assay (C). All testing was carried out in triplicate, on three separate occasions. Data represents mean ± SD, statistical analysis compared untreated to treated biofilms (*p<0.05, **p<0.01, ***p<0.001). #Indicates no cell growth.

Figure 5 - Molecular analysis demonstrates a significant microbial burden with limited activity of CHX on 3-D biofilms. Bacterial and fungal monospecies biofilms were grown in the 3-D model, as previously described. Following development, biofilms were washed and treated with PVP-I (10%) or CHX (0.05%) for 24 h. Live/Dead PCR was performed and colony-forming equivalents (CFE) were calculated from standard curves for *C. albicans* (A), *S. aureus* (B) and *P. aeruginosa* (C). Data represents mean ± SD. * Represents statistical difference in total CFE values and # represents significant differences between live CFE values (*p<0.05, **p<0.01, ***p<0.001). Data represents CFE values calculated from triplicates carried out on three separate occasions.

Figure 6 – Molecular analysis show compositional changes after treatment, especially with PVP-I, and SEM confirms high levels of growth in the triadic 3-D model. Bacterial and fungal triadic biofilms were grown in the 3-D model, as previously described. Following development, biofilms were washed and treated with PVP-I (10%) or CHX (0.05%) for 24 h. Live/Dead PCR was performed and colony-forming equivalents (CFE) were calculated from standard curves for untreated (A), PVP-I (B) and CHX (C). Data represents percentage composition calculated from CFE values from triplicates carried out on three separate occasions. SEM, shown in lower panels, was conducted as described in the methods. Note *P. aeruginosa* indicated by a solid white arrow, *S. aureus* by white arrow head, *C. albicans* by a black arrow. In the 3-D model, *C. albicans* is seen as mostly yeast; here bacteria and yeast are seen in clusters upon the cellulose matrix. Bars represent 20 µm on lower magnifications (×1000) and 2 µm at higher magnification (inset, ×6000).

Figure 7 – Principal Component Analysis shows little effect of CHX treatment on cellulose matrix biofilms, whilst treatment of the 2-D model caused a shift to new clusters. PCA reduces the dimensionality of the data to form clusters. The axes represent the two principal components of the data which showed the highest variance. These cluster patterns showed treatment with CHX does not impact cellulose matrix biofilms, and PVP-I caused a similar compositional change in the 3-D model to that of

1 CHX treatment in the 2-D model. ● Untreated 2-D, + Untreated 3-D, ▫ PVP-I 2-D, ▪ PVP-
2 I 3-D, × CHX 2-D,○ CHX 3-D.